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(54) Title: TRANSFORMING GROWTH FACTOR (TFG) β SUPERFAMILY ANTAGONISTS

(57) Abstract

Antagonists for biological processes induced by dimers or multimers are described that are easily derivable from the respective dimers or multimers by omission of at least one nonomer unit and/or at least one wrongly bound or folded nonomer unit. Particularly suitable antagonists of this kind are BMP antagonists that are a very efficient agent against undesired ossification due to trauma or operations such as hip replacement.

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Transforming Growth Factor (TFG) β Superfamily Antagonists

Technical Field

The present invention concerns antagonists against diseases induced by agonists needing for their activation more than one receptor-agonist interaction. In particular the present invention concerns antagonists for cytokines, preferably of the transforming growth factor(TGF) β superfamily.

Background Art

It is known that several biologically active molecules, below referred to as agonists, are dimers or multimers (trimers, tetramers, etc.) and need for their activation the simultaneous interaction with two or more receptor sites of the same or different receptors.

A well known family of such agonists are the cytokines, in particular the transforming growth factor(TGF) β superfamily. Such factors are e.g. involved in heterotopic ossification (HO).

Heterotopic ossification (HO) is a normal bone formation at ectopic sites like muscle and connective tissue, that can lead to a decreased range of motion, pain, or even total ankylosis predominantly of hip or elbow joints (see (6, 14)). In contrast, orthotopic ossifications is characterised by normal bone formation contiguous with the normal skeleton. HO may occur due to genetic disorders, but it is commonest following surgical trauma especially total hip replacement or head and neck trauma.

Although the mechanisms that cause HO after trauma are not entirely understood, one key player of all ossifications are certainly bone morphogenetic proteins (BMPs). They are member of the TGF β superfamily and are

able to induce heterotopic bone formation (33,34). Like TGF β, BMPs are synthesised as precursor molecule, which dimerise and get glycosylated (32). The majority of the BMP protein is cleaved at a basic peptide sequence, yielding the active, mature BMP molecule as a disulfide dimer of the carboxy-terminal quarter of the proprotein. Mature BMPs appear as a broad band around 30 kDa on SDS-PAGE, and reduce to monomers in the range of 16-20 kDa.

BMPs, or bone morphogenetic proteins are well described (1, 19, 26-29, 34). Until now more than 30 different BMP-like proteins are known (for review see (20)). Together they form the BMP family which comprises all BMPs, all OPs (osteogenic protein), CDMPs (cartilage-derived morphogenetic protein), GDFs (growth/differentiation factor), Dpp (decapentaplegic) and Vg (vegetal). The BMP family belongs to a larger family known as the transforming growth factor β (TGF β) superfamily, which includes besides TGF βs, also activins/inhibins, and Móllerian inhibiting substances (35). Analogous to other members of the TGF β superfamily the BMP receptors I and II are serine/threonine kinases. Upon BMP binding, BMP receptor I and II form a heteromeric-activated receptor complex, which initiates the signal transduction cascade (20) and leads to the activation of different genes involved in osteogenesis. The prerequest for the induction of osteogenesis by BMPs is the presence of cells with BMP receptors present in their cell membrane. Only these cells are able to receive BMP signals and respond to them. For the occurrence of the phenomena of heterotopic ossification, especially after total hip replacement, Friedenstein (7) and Owen (17,18) postulated the existence of two osteogenic precursor cells in the periarticular tissue of the hip. The inducible progenitor cells need BMP to develop into osteoblasts. They are located in the periarticular soft tissue and migrate and circulate in the blood stream. The second type called determined osteogenic progenitor cells originate in stromal parent

cells of the bone marrow and develop into osteoblasts upon contact with non-resident tissue. Although they don't need BMPs for the induction of bone formation, they respond to BMPs with an accelerated differentiation (21). After the induction of bone formation, the bone formation itself is influenced by BMPs, because all osteoblasts respond to BMP signals (8,22) by a stimulation of proliferation and differentiation.

Besides BMPs there are other effectors on bone formation, which were shown to promote the osteogenic activity of BMPs. Most of them are cytokines but also prostaglandins are known to enhance bone formation and to promote the activity of BMPs (16). A decrease of prostaglandins in patients treated with non-steroidal anti-inflammatory drugs (NSAIDs) is the reason for a decrease in the number of patients developing HO after total hip replacement (6). In 1975 Dahl (3) was the first to demonstrate that NSAIDs are useful for the prophylaxis against HO. He used indomethacin, but also ibuprofen, acetylsalicylic acid, and diclofenac have been investigated in the following years (10) and shown to reduce the occurrence of HO. The treatment has to start just before or immediately after operation and should be continued for at least 8 days (5).

In 1981 postoperative radiation in preventing HO was introduced by Coventry and Scanlon (2) This treatment aims directly at the osteogenic precursors (6) and inhibits their differentiation to osteoblasts. If radiation therapy is used to reduce the occurrence of HO, it is sufficient to apply 1x7 Gy preoperatively or 17.5 Gy postoperatively if administered not later than 96 hours after hip replacement 2.

The state of the art treatments bear a lot of disadvantages. About 30% of patients treated with NSAIDs develop side effects like: pyrexia, allergic reaction, gastrointestinal discomfort, gastric ulceration or central nervous effects (10) and the osseointegration of the

implant is retarded. The length and onset of the treatment with NSAIDs is still in dispute, but in animal studies it was shown that indomethacin could only prevent demineralized bone-induced heterotopic ossification if administered 6 hours before the implantation (4). Thus, HO induced through accidents, like head and neck traumas, can not be treated to date.

Compared to the treatment with NSAIDs radiotherapy is very cost intensive. Other disadvantages of radiotherapy are linked to the overall deterious effects of radiation, like the induction of transient oligospermia or infertility and initiation of secondary malignancies. Radiation can even be deterious for the osseointegration of alloplastic implants and reduce short- and long-term stability of the prosthesis (9,31) Also cancer patients which underwent radiation therapy should not be exposed to additional radiation and are therefore excluded from HO prophylaxis by radiation therapy.

Ossifications can also be a manifestation of inherited and acquired bone forming lesions. Here the only possible treatment is the surgical removal of the new formed bone. But this could prove to be very difficult and dangerous like in spinal hyperostosis or myelopathy caused by the ossification of the posterior longitudinal ligament in the cervical spine (12) which leads to spinal cord compressions . Not operable is Spondylitis ankylosans (Bechterew-Strümpel-Marie-Krankheit) where ossification is linked to inflammation and possible auto immune response triggered by Klebsiella antigens (23). The main problem for all different ossifications is that even if the extra bone can be removed by surgery the trigger for ossifications still exists and recurrence will take place. Thus, it is very much desirable that a successful removal of the extra bone by surgery is accompanied by an efficient inhibition of ossification at the operation site, whereby such inhibition should be almost free of undesired side effects.

Operations to reduce extra bone can also be performed in the case of cranio-metaphyseal dysplasia. But in the inherited diseases myositis ossificans and fibrodysplasia ossificans progressiva no treatment can be applied, due to the fact that any treatment tested so far led to severe progression of the disease (25,36).

Known antagonists are e.g. receptor mimics such as fetuin or neutralizing anti-TGF-beta antibodies that block osteogenesis (37), IL-2 and IL-6 variants carrying specific substitutions (38 and 39), RANTES extended by addition of one amino acid at its N-terminus (40), and soluble receptors, namely the interleukin-4-receptor (41).

However, to find and/or generate such antagonists is rather time consuming and often limited to good knowledge of the receptors and agonists as well as their interactions or interacting sites, respectively.

It is thus still very much desired to get products to inhibit unwanted biological effects, such as for example ossification, that are efficient, poor in or free of undesired side effects, and, preferably, also effective soon after administration, and that preferably are readily available.

Disclosure of the invention

The present invention concerns an antagonist for biological processes induced by a dimer or a multimer activating such processes due to interactions with more than one receptor site, which antagonist is characterised in that it interacts with at least one first receptor site needed to activate the biological process and in that it does not interact with at least one second receptor site needed for such activation, and whereby said interaction with said second receptor site does not take place due to at least one monomer unit of said dimer or

multimer being missing or folded thus that a biological process activating interaction with said second receptor site is impossible. Such antagonists either lack at least one monomer unit or have one of their monomer units folded thus that no interaction is possible.

The terms "folded", "folding" etc. as they are used in the scope of the present invention comprise any conformation of dimers or multimers wherein at least one monomer unit is differently positioned relative to at least one further monomer unit if compared with the conformation of the respective agonist. Such folding can actually be due to a different folding, but also to a wrong binding of one of the monomer units, e.g. at binding at a wrong dimerisation site.

Preferred antagonists are antagonists to cytokines, such as for example TGF β, interleukin-5, interleukin-6, interleukin-10, interleukin-12, hepatocyte growth factor, platelet derived growth factor, and macrophage-colony stimulating factor. In particular preferred are antagonists to members of the TGF-β superfamily, especially antagonists to members of the BMP superfamily.

Antagonists lacking at least one monomer unit or consisting of one monomer unit can be produced in that a host cell is transformed with a DNA sequence encoding the respective agonist and cultured under conditions allowing the expression of the monomer units building up said agonist, and in that the product of the expression is solubilized and treated under non-oxidising or reductive conditions. If only a part of a monomer unit is desired, a monomer unit produced as described above can be treated by well known methods to reduce the size of amino acid sequences. Alternatively it is of course also possible to produce such antagonists by expression of a DNA encoding such a modifies monomer unit in a suitable host cell.

Antagonist acting due to folding can be produced in that a host cell is transformed with a DNA se-

quence encoding the respective agonist and cultured under conditions allowing the expression of the monomer units building up said agonist, and in that the product of the expression is solubilized and treated under oxidising conditions.

Antagonists of the present invention do also comprise such antagonists wherein the at least one lacking interaction with at least one receptor site does not take place due to at least one monomer unit of said dimer or multimer being folded thus that a biological process activating interaction with said second receptor site is impossible, due to an extension of the amino acid sequence of one of said monomer units at its N-terminal end by at least 5, preferably 10 to 30 amino acids, e.g. the sequence N-Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met-C. Also such an antagonist is preferably an antagonist to members of the TGF-β superfamily , in particular an antagonist to members of the BMP superfamily.

The present invention also concerns a DNA sequence encoding BMP that is extended at its N-terminus by a sequence as defined above.

An antagonist folded due to an extension at its N-terminus can be produced

- in that a DNA sequence encoding the respective dimer or multimer is extended at its N-terminus by a sequence according to claim 12 either prior to its introduction into a suitable vector or by the introduction into a vector comprising such an extension,
- in that a suitable host cell is transfected with said vector and cultured under conditions allowing the expression of the antagonist or monomer units of said antagonist, and
- in that the product of the expression of said DNA sequence is solubilised and treated under oxidising conditions.

The antagonist of the present invention are very valuable effective substances in the treatment of a broad variety of dimer or multimer induced diseases. In particular antagonists to members of the BMP superfamily are very valuable agents against heterotopic ossification (HO).

Modes for carrying out the invention

The present invention provides specific antagonists and a method for the production of antagonists for biological processes induced by dimers or multimers activating such processes due to multiple interactions with receptor sites located within a specific distance, usually on one cell. Said antagonists are characterised in that they interact with or bond to at least one site of at least one receptor needed to activate the corresponding agonist and leave at least one further reception site free, whereby at least one of the monomeric units is cleaved off or wrongly bound thus that an activating interaction with at least one receptor site is impossible due to a change in the localisation of at least one binding site relative to another binding site.

The inventive antagonists for homodimers thus are monomers or parts of monomers or wrongly connected dimers. Also for heterodimers the inventive antagonists can be monomers or parts of monomers, whereby in the case of a preferred first receptor site the monomer unit binding to said preferred first binding site is the preferred monomer. If the preferred receptor site is not known or if no great difference in the binding speed is present, a mixture of monomers might be used. Furthermore also heterodimers with a wrongly bound unit are suitable, since they provide both interacting sites but in a configuration allowing only one of the two possible (and for an agonist activity needed) interactions. The same applies for multimers with three or more subunits of the same or

different kind. Antagonists to such multimers comprise monomers with the preferences discussed with regard to the heterodimers, as well as dimers or multimers provided that they lack at least one monomer unit or have at least one monomer unit wrongly bound so that at least one interaction needed for agonist activity lacks. Furthermore also for heterodimers and multimers parts of a monomer unit can be used as antagonist provided that they have a sufficiently fast and strong interaction with one of the first binding receptor sites.

It was surprisingly found that the antagonists of the present invention readily bind at least one receptor site and are not replaced by the agonist although the agonist has two or more binding sites so that a preference of the correctly binding agonist had been expected, the more so since the steric effect of at least a monomer or a binding part of a monomer is much reduced over a dimer or multimer.

In particular the present invention concerns antagonists to cytokines, such as for example TGF β, interleukin-5, interleukin-6 (multimer), interleukin-10, interleukin-12, hepatocyte growth factor, platelet derived growth factor, and macrophage-colony stimulating factor. A preferred group of antagonists are those to members of the TGF-β superfamily, and as ossification antagonists those to the BMP superfamily are much preferred. Members of the TGF-β superfamily, for example, with the cytokines being homodimers or heterodimers and the signal transduction being initiated by a cytokine mediated dimerisation of two receptors upon cytokine binding are especially suitable for the generation of antagonists according to the present invention.

Especially the invention comprises the production and/or folding of antagonists for cytokines, preferably members of the TGF-β superfamily, especially members of the BMP-superfamily, as well as respective antagonists.

The agonists suitable for deriving therefrom the antagonists of the present invention also include specific mutants of agonists needing for being active the simultaneous interaction with at least two receptor sites, in particular mutants of cytokines, and very much preferred specific mutants of bone morphogenetic proteins with improved refolding properties. From such agonists monomeric and dimeric antagonists lacking at least one "possibility" for simultaneous interaction easily derivable. The "possibility" for simultaneous interaction means the presence of a correctly placed binding site, the lack of such possibility either the lack of such a binding site (absence of at least one monomer unit) or a wrongly situated binding site. Such polypeptides are obtainable by a method that is also an object of this invention, and in general such polypeptides are obtained in high yields.

In accordance with the present invention and as specific embodiments thereof, mutant forms or native forms of recombinant bone morphogenetic proteins 2, 4 and 7 and other BMP-like proteins may be used to produce large quantities of BMP monomers homo- or heterodimers from bacteria that are folded into biologically active dimer or monomer molecules acting as BMP-antagonists.

As already defined above, folded refers to the antagonists with at least one monomer unit wrongly folded, e.g. due to its binding to a wrong dimerisation site. In contrary, the term refolding refers to the conformation of the polypeptide associated with the natural biological activity and includes the dimerisation.

The molecules of the present invention also include DNA molecules comprising a nucleotide sequence encoding BMP-2,-4 and -7 except that the N-terminus was extended by some amino acids, preferably about 21 amino acids. The extension is based on the idea, that in nature all BMPs are synthesised first as pro-peptides and therefore the folding of the C-terminus, which finally forms

the mature BMP, might be influenced by a N-terminal extension. As DNA source which codes for the 21 amino acid-extension any suitable vector can be used such as the commercially available vector pET-28-a from Novagen. The original mature DNA sequence for BMPs can be mutagenized by PCR and a Nde-restriction site can be created at the N-terminus. With such an Nde restriction site it is easy to place the mutagenized DNA for BMPs into e.g. the above mentioned vector. Such modifications are well within the level of ordinary skill in the art. The N-terminal extension of the protein produced e.g. in said specific vector is: N-Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met-C. But other extensions may be used to influence the folding of the mature BMP and are also covered by this invention.

Nucleotide sequences encoding no N-terminal extension can also be cloned into a commercially available vector such as e.g. pET-23-a, from Novagen using the same Nde-restriction site as stated earlier. But any bacterial expression vector may be used, as long as it is capable of directing the expression of a heterologous protein such as BMP in the bacteria chosen. The modification including the extension are well within the level of ordinary skill in the art.

The bacterial expression plasmid may be transformed into a competent bacterial cell using known methods. Transformants are selected for growth on medium containing an appropriate drug, when drug resistance is used as the selective pressure. For the production of recombinant BMP any bacterial species may be used.

The BMP expressed in such transformed bacteria cells is present in inclusion bodies, which are aggregates of precipitated BMP monomers and can be isolated from disrupted cells by centrifugation. The inclusion bodies can be solubilized by acidification with acetic acid or trifluoroacetic acid and reduced by a reducing agent such as ß-mercaptoethanol or dithiotreitol. The pH

for solubilization and denaturation proved to be preferably between 2 to about 4, but also basic conditions with pH above 10 can be used. The inclusion bodies can also be dissolved in chaotropic agents, well known in the art, like urea or guanidine hydrochloride. The concentration of chaotropic agents are normally in the range between 4 to 9 M.

The goal of all work known in the art on the refolding of proteins especially of TGF-like proteins is aimed on the recovery of biological active TGF-like proteins, with the same function as the TGF-like proteins in nature. In contrary, the goal of the present invention is to receive folded TGF-like proteins which antagonise the natural action of TGF-like proteins. Based on the knowledge that in order to transduce the signal of TGF-like proteins, homodimers or heterodimers have to bind two receptors to form a heteromeric-activated receptor complex, possible antagonist have to bind receptors and block the formation of heteromeric activated receptor complexes.

As example to show the efficacy of the antagonists of the present invention BMPs were chosen, due to the fact that natural BMPs induce bone formation and BMP-antagonists should inhibit bone formation. Both actions can be tested in vivo, using demineralized bone matrix as carrier to determine the effect as antagonist and inactive collagenous bone matrix as carrier to determine the natural action of BMPs (13). As parameter for ossification the amount of calcium per mg implant can be used, as well as the extent of ossification judged on the basis of toluidin-blue stained or Goldner stained histosections. All these measurements and histo-staining are well within the ordinary skill in the art.

As one type of antagonists for natural actions of BMPs, BMP monomers were synthesised, which bind BMP-receptors without leading to the formation of activated receptor complexes. For the production of monomers the same procedures as described in US patent 5756308 or

EP 0433225 A1 can be used. Preferably the solubilization of BMP-monomer inclusion bodies is performed with urea without reducing agents. A preferred solubilization buffer contains: 6M urea, 20 mM Tris-HCl pH 7,9; 0,5 M NaCl; 5 mM imidazole. The solubilization could be shown to be complete after 4-6 days at 4°C under agitation at high protein concentrations (1-5 mg/ml). The solubilized protein can be further purified using known chromatographic methods such as size exclusion chromatography, or exchange chromatography, or reverse phase high performance liquid chromatography. As last purification step gel filtration is preferably used to separate monomers from dimer or oligomers. The final gel filtration step is preferably performed in a buffer containing 6M urea and 25 mM Tris-HCl pH 8, but other buffers with chaotropic salts serve the same purpose.

This invention also comprises the formation of dimers, preferably BMP-dimers, which are folded to antagonise the natural action of BMPs. That BMP-dimers acting as antagonists can easily be obtained is due to the fact that in each mature BMP-monomer 6 cysteins are present, which represent potential dimerisation sites. In nature only specific cysteins are involved in dimerisation. Thus, if no reducing agent is used during the solubilization process, a substantial amount of monomers is oxidised to dimers. The oxidation is due to the presence of air or can be favoured by the use of oxidising agents, like glutathione. If choatropic or other denaturing agents are present during the dimerisation or if the protein concentration is high, dimerisation can occur at unnatural sites and thus effect the 3 dimensional overall structure of the dimer. This can lead to products where one or both halves can bind to receptors, but the formation of a heteromeric activated receptor complex is hindered due to steric reasons caused by the change of the overall 3-dimensional structure of the dimer.

Inhibiting monomers can also be produced by reducing bioactive BMP-dimers with for example ß-mercaptoethanol or dithiotreitol. As source for bioactive BMP-dimers, refolded bacterial BMP, BMPs produced in mammalian cell lines like CHO-cells or BHK cells, or natural BMPs isolated from animals like bovines, or other species can be used.

As already mentioned above, the present invention is not restricted to BMPs or BMP-like proteins, but covers also other cytokines which have to bind two receptors for the transduction of the signal like other members of the TGF-superfamily. In addition, possible mutants of cytokines and random polypeptides which bind cytokine receptors and antagonise the action of cytokines are also covered.

By virtue of the invention it is possible to obtain polypeptides for use in the prevention of cytokine actions requiring simultaneous interaction with at least two receptor sites, such as ossifications including heterotopic ossifications and other diseases linked to ossifications.

Heterotopic ossification and ankylosis due to HO can be inhibited locally by applying antagonists of the present invention during the operation at the operated hip or directly at the most likely affected knee or elbow-joints. In order to prevent heterotopic ossification induced by head and neck traumas or other traumatic incidences, the invention can be applied directly at knee, hip, and elbow joints, where ankylosis is most dramatic and most likely to occur. The advantage of the antagonists of the present invention in comparison to NSAIDs and radiotherapy is, that they are more specific and therefore much reduced in, or even without side effects. Therefore a routine prophylaxis treatment with antagonists of the present invention is justified after all hip replacements. In addition, such antagonists can also be applied shortly after the heterotopic ossifications

were induced. Thus patients which suffer from head and neck traumas or other accidental traumas could also undergo a routine HO prophylaxis treatment. With the arising availability and use of human recombinant BMPs for the initiation of ossification, antagonists of the present invention can also serve to restrict the ossification to a certain region or to stop ossification induced by human recombinant BMPs.

The present invention also opens a new field of treating diseases, linked to bone formation. For the treatment of inherited or induced bone forming lesions like heterotopic ossification, spinal hyperostosis, spondylitis ankylosans, cranio-metaphyseal dysplasia, myositis ossificans, or fibrodysplasia ossificans progressiva antagonists of the present invention could be administered at the operation site in order to inhibit further ossification, or the recurrence of HO. In the case that operations can not be performed, the inventive antagonists can be applied by injections in the regions where ossification occurs, or applied systemically. In the case of fibrodysplasia ossificans progressiva, where any injection leads to new ossifications, an inventive antagonist is preferably administered systemically. If injections in such patients are mandatory, the inventive antagonists with or without carrier can be mixed with the medication in order to inhibit bone formation at the injection site.

Vascular invasion is a prerequest for bone formation. The very early and transient increase in BMP-4 mRNA during fracture healing (43, 44) and its decisive role in hematopoiesis (42) indicate a crucial role of BMP-4 especially for the blood supply during bone formation. Vascularisation is a critical point for the ossification but it is also important for cancer development and tumor growth, because fast growing tumors need a lot of nutrients. In the last years, the efficacy of inhibitors for angiogenesis in stopping the growth of tumors

were shown. This is a further field of application of antagonists of the present invention such as BMP-4 antagonists. Thus BMP-antagonists, in particular BMP-4-antagonists, additionally represent a new class of tumor suppressors.

Still another field of application of an inhibitor for vascularization such as a BMP-4-antagonist is in the first non invasive treatment for hemagiomas and vascular malformations. Severe formes of these diseaeses are very difficult to treat and can even be deadly. Especially relapses after surgical removal of hemangiomas or after embolization are very frequent and can be avoided by the use of an inhibitor for vascularisation.

Antagonists of the present invention can be applied alone, i.e. as single inhibitor, or in combination with other antagonists of the present invention or known antagonists, as a mixture of inhibitors. The application can be performed locally in an aqueous solution in combination with a carrier like collagen or entrapped in a biodegradable material, for example polylactide-co-glycolide acids microspheres as described for the application of BMP dimers (30). The composition of the later one is suitable to determine the release of antagonists of the present invention to the target sites. Systemic application can be achieved by a formulation as liquid, pill, tablet, lozenges for enteral administration, or in liquid form for parenteral administration.

Examples

Example 1: Solubilization and folding of BMPs in E.coli

The expression of the monomers was performed according the recommendations of Novagen from which the E.coli strain (BL21) and the different pET-vectors were bought. The cells in which the BMP monomers were ex-

pressed, were harvested by centrifugation at 5000xg and frozen at -80°C.

6,2 grams of frozen cell pellet were thawed and resuspended in 40 ml Buffer 1 (20mM Tris-HCl pH 7,9; 0,5M NaCl; 5mM imidazole). Cells were disrupted by 2 passes through a French press. The lysate was diluted to 50 ml and centrifuged at 15000xg for 15 minutes. The pellet formed by inclusion bodies was resuspended in buffer 1 with the help of a dounce homogenisator and centrifuged again at 15000xg for 15 minutes. The supernatant was discarded and the pellet homogenised with a dounce homogenisator, in 50 ml of Buffer1+6M Urea. The volume of the final homogenate was adjusted to 50 ml and rotated on a turning wheel for 6 days at 4°C. The resuspended inclusion bodies were centrifuged for 20 minutes at 10000xg, the pellet discarded and the supernatant filtered through a sterile filter from Nalgene with 0.8 µm pores size.

If a BMP mutant with the 21 additional amino acids N-Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met-C was produced, the protein was purified by a nickel affinity column. This is possible because the 21 amino acid N-terminal extension contains a stretch of 6 histidines. As affinity resin the Ni-NTA Superflow™ (Quiagen) was used. The column size was 1,6cmx10cm (20 ml). 75- 100 mg of protein in 30-50 ml Buffer1+6M urea were loaded onto the column at 2 ml/min. After a wash step with 50 ml of [8M urea; 0,1M NaH2PO4; 10mM Tris -HCl pH 6.3] and another one with 75 ml [8M urea; 0.1M NaH2PO4; 10 mM Tris-HCl pH 4.5] the protein was eluted with 75 ml [20mM Tris-HCl pH 7,9; 0.5M NaCl; 1M imidazole].

Before the purification could be continued, the protein solution (30 ml) was dialysed with a size exclusion of 10000 MW against 2x1000 ml TU (25 mM Tris pH 8.0; 6 M urea] over night. After the dialysation the protein solution was centrifuged at 5000xg for 15 minutes

and sterile filtered with a filter of 0.45 µm pore size. Alternatively the protein solution was concentrated with an Ultrafree® Biomax-10K from Millipore and the buffer exchanged by 0.5M arginine; 10mM histidine pH 6.3. The protein precipitated in this buffer and was centrifuged at 15000xg for 10 minutes. The supernatant was discarded and the pellet dissolved in TU. With a further centrifugation step at 15000xg for 10 minutes insoluble material was pelleted and discarded. Alternatively the protein solution was concentrated with a 15 ml Ultrafree® Biomax-10K from Millipore and the buffer exchanged by several passes of 10x the volume by TU. The protein solution in TU was centrifuged at 15000xg for 10 minutes and the supernatant was used for further purification steps.

All the mentioned approaches finished with the protein being dissolved in TU. Now it could be applied to a Heparin column at 2 ml/min (5ml HiTrap® from Pharmacia Biotech) in batches of 20 mg, washed at 5ml/min with 25 ml TU, and eluted by a step gradient with TU+2M NaCl at the same speed. Alternatively the Heparin column was made of Heparin-Sepharose CL-6B (Pharmacia Biotech). In this case all steps were performed at 2 ml/min. The dimension of the column was 2.6cm x15 cm (80ml).

For gel filtration 2ml of the protein eluted from the Heparin columns were loaded with 2-15 mg on either a Hiload® Superdex® 75 or 200 prep grade column from Pharmacia Biotech (1,6 cm x 60 cm; 124 ml). On this columns the oligomer, dimer, and monomer of all different BMPs or mutagenized BMPs could be separated. 1 ml fractions were collected and the column was run with TU. Fractions, which only contained dimers or monomers were pooled. The decision on which fractions to pool was made based on the result of SDS-PAGE under non reducing conditions. If the protein content of the pools was below 0.4 mg/ml it was concentrated with a 15 ml Ultrafree® Biomax-10K from Millipore to about 0.5 to 2 mg/ml. Protein con-

centrations were determined by "Coomassie® Plus Protein Assay Reagent" from Pierce.

Example 2: Determination of ossification and it's inhibition

The extent and potency to induce or inhibit ossification were determined in vivo in rats. Inactivated bone collagen matrix (IBCM) and demineralized bone (DB) were produced as described in (13) and used for carrier. These methods are within the normal scope of the skill of the art. The protein solution was added to the carrier in TU or in 5mM HCl. In the latter case buffer was exchanged with 5mM HCl by 3x1:10 concentration steps with a 15 ml Ultrafree® Biomax-10K from Millipore.

25 mg of carrier in an eppendorf®-tube was mixed with 120µl antagonist probe in 5mM HCl or TU containing 0.5 or 1 mg chondroitin 6-sulfate sodium from FLUKA. In the control probe no antagonist was added. The loading took place at RT. After 1h, 300µl of collagen (Collagen R, 2mg/ml in 0.1% acetic acid from Serva) was added and mixed with the implant material. The proteins were precipitated by the addition of 1.2 ml 100% EtOH (pre-cooled -80°C) and the material transferred to -80°C. After 1 h in the freezer the implant material was centrifuged at 15000xg 15 min at 4°C, the supernatant discarded and replaced by 80% EtOH (-20°C). After another centrifugation for 5 min at 15000xg the entire supernatant was removed and pellets were formed using a 1 ml syringe. The implants were dried in a hood over night.

At the next morning rats were anaesthetised and the probes implanted either subcutaneously or intramuscularly at bilateral sites over the thorax. After 23-28 days the rats were killed by CO_2 and the probes removed. The explant was freed from adherent tissue and cut

in half. One half was used for histo-chemistry, the other half was weighed and homogenised in 1,5 ml of cold 3mM sodium-bicarbonate buffered saline, pH 9. The homogenate was centrifuged at 7500xg for 15 minutes. In the supernatant the alkaline phosphatase activity could be determined by the department of clinical chemistry of the UniversitätsSpital Zurich. The pellet was resuspended in 1 ml 5mM Tris-HCl pH 7.2, stirred for 1h, and centrifuged for 15 min at 15000xg. This wash procedure was repeated 3 times. To the final pellet 1ml of 0.5M HCl was added and stirred over night. After another centrifugation (15000xg; 15 minutes) the supernatant was given to the department of clinical chemistry of the UniversitätsSpital Zurich for the determination of the calcium concentration by atomic absorption spectrophotometry. The values for calcium concentration and the weight of the half of the implant determined the calcium content of the implant expressed in [mgCa/gr Implant] (see Example 3).

Example 3: Test of inventive agonists

In the first set of experiments the 21 amino acid extended BMP2(=B2mat, BMP4(=B4mat), or BMP-7(=B7mat) were expressed in E.coli and purified by a nickel-affinity column and heparin column as indicated earlier. In this preparation monomers and dimers were present. As carrier IBCM was used and up to 50µg BMP applied. After 21 days in the rat, none of the implants showed an increase in calcium.

In the next experiment the monomers and dimers of B4mat were separated by a gel filtration step. 30µg dimers were loaded on IBCM. After 21 day in the rat, one out of 3 probes showed an increase in calcium by the factor of 5. The experiment was repeated in 4 animals with 60µg B4mat dimer. After 26 days only in one animal

no difference between the calcium content of the control and protein loaded was seen. In 3 animals the calcium content in comparison to the control implant was increased by the factor 1.2, 2.6, and 18.5. From these experiments we concluded that the monomer present in monomer-dimer mixtures can inhibit the action of BMP-dimers.

In order to test the hypothesis that BMP-monomers can inhibit heterotopic ossification, we used demineralized bone as carrier. If DB is implanted at ectopic sites it leads to heterotopic bone formation. This animal model is one of the rare models for heterotopic ossification(15) and was also used to show the efficiency of NSAIDs and radiotherapy for the prevention of HO. In 4 experiments with rats of different age, 3 independent monomer-preparations (normal B4; B4mat (=21 amino acids extended B4) the hypothesis could be confirmed:

Results:

	mg Ca/g implant with antagonist/ control [%]					
BMP-monomers	Days	No	mean	SD	site	independent preparation
12µg B4mat	26	3	14	4.9	i.m.	+
50µg B4	23	3	67	32	s.c.	+
60µg B4	25	3	31	14	s.c.	
50µg B4mat	27	4	72	7.5	s.c.	+
		13	48	30		

The results on calcium content were confirmed by the Goldner and toluidin blue stained histosections. Without antagonists the ossification of the implant was advanced in comparison to implants with BMP-monomers. The results clearly show, that the monomer from BMP-4 with and without N-terminal extension inhibit or retard the ossification.

In comparison DiCesare and co-workers (4) showed that after 28 days indomethacin inhibited the demineralized bone-induced heterotopic ossification expressed in calcium content to 21% of the control value, but only if the treatment was started at least 6 h before implantation. After implantation indomethacin was administered on daily basis.

In our treatment with BMP-monomers the treatment started at the time point of implantation and the monomers were only applied once. Thus, an administration with a slow release device could be a more efficient prophylactics for heterotopic ossification.

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

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Claims

1. An antagonist for biological processes induced by a dimer or a multimer activating such processes due to interactions with more than one receptor site, characterised in that said antagonist interacts with at least one first receptor site needed to activate the biological process and does not interact with at least one second receptor site needed for such activation, and whereby said interaction with said second receptor site does not take place due to at least one monomer unit of said dimer or multimer being missing or folded thus that a biological process activating interaction with said second receptor site is impossible.

2. The antagonist of claim 1 that lacks at least one monomer unit.

3. The antagonist of claim 1 or 2 wherein one of its monomer units is folded.

4. The antagonist of anyone of claims 1 to 3 which is an antagonist to cytokines, such as for example TGF β, interleukin-5, interleukin-6, interleukin-10, interleukin-12, hepatocyte growth factor, platelet derived growth factor, and macrophage-colony stimulating factor.

5. The antagonist of anyone of claims 1 to 3 which is an antagonist to members of the TGF-β superfamily, in particular an antagonist to members of the BMP superfamily.

6. A method for the production of the antagonist of claim 2, characterised in that a host cell is transformed with a DNA sequence encoding the respective agonist and cultured under conditions allowing the expression of the monomer units building up said agonist, and in that the product of the expression is solubilized and treated under non-oxidising or reductive conditions.

7. A method for the production of the antagonist of claim 3, characterised in that a host cell is transformed with a DNA sequence encoding the respective

agonist and cultured under conditions allowing the expression of the monomer units building up said agonist, and in that the product of the expression is solubilized and treated under oxidising conditions.

8. An antagonist for biological processes induced by a dimer or a multimer activating such processes due to interactions with more than one receptor site, characterised in that said antagonist interacts with at least one first receptor site needed to activate the biological process and does not interact with at least one second receptor site needed for such activation, and whereby said interaction with said second receptor site does not take place due to at least one monomer unit of said dimer or multimer being folded thus that a biological process activating interaction with said second receptor site is impossible, due to an extension of the amino acid sequence of one of said monomer units at its N-terminal end by at least 5 amino acids.

9. The antagonist of claim 8 wherein the extension has a length of 10 to 30 amino acids.

10. The antagonist of claim 8 or 9 wherein the extension has the sequence

N-Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met-C .

11. The antagonist of anyone of claims 8 to 10 which is an antagonist to members of the TGF-β superfamily , in particular an antagonist to members of the BMP superfamily.

12. A DNA encoding BMP that is extended at its N-terminus by a sequence of at least 5, preferably 10 to 30 amino acids, in particular the sequence

N-Met-Gly-Ser-Ser-His-His-His-His-His-His-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met-C.

13. A method for the production of an antagonist of anyone of claims 8 to 11, characterised

- in that a DNA sequence encoding the respective dimer or multimer is extended at its N-terminus by a

sequence according to claim 12 either prior to its introduction into a suitable vector or by the introduction into a vector comprising such an extension,

- in that a suitable host cell is transfected with said vector and cultured under conditions allowing the expression of the antagonist or monomer units of said antagonist, and

- in that the product of the expression of said DNA sequence is solubilised and treated under oxidising conditions.

14. The antagonist of anyone of claims 1 to 5 and 8 to 11 as effective substance in the treatment of diseases.

15. Use of an antagonist of anyone of claims 4, 5 or 11 for the preparation of a pharmaceutical against heterotopic ossification (HO).

16. A pharmaceutical composition comprising an antagonist according to anyone of claims 1 to 5 and 8 to 11.

17. A pharmaceutical composition comprising an antagonist according to anyone of claims 4, 5 or 11 for the treatment of heterotopic ossification (HO).

SEQUENCE LISTING

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<120> Transforming Growth Factor(TGF) beta Superfamily Antagonists

<130> 02302PC Universität Zürich

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: N-terminal amino acid extension influencing the dimer or multimer folding

<400> 1

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Arg Gly Ser His Met
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INTERNATIONAL SEARCH REPORT

Intern. ..onal Application No
PCT/IB 99/00466

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 C07K14/51 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 17935 A (GLAXO) 13 June 1996 (1996-06-13) the whole document	1-17
A	EP 0 881 288 A (HOECHST AG) 2 December 1998 (1998-12-02) the whole document	10

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 January 2000	25/01/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Masturzo, P

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INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/IB 99/00466

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BIOLOGICAL ABSTRACTS, vol. 1998, 1998 Philadelphia, PA, US; abstract no. 121085, F S KAPLAN & E M SHORE: "Encrypted morphogens of skeletogenesis; biological errors and pharmacological potential" XP002124560 & BIOCHEMICAL PHARMACOLOGY, vol. 55, no. 4, 15 February 1998 (1998-02-15), pages 373-382, OXFORD, GB ISSN: 0006-2952 abstract	1-17
A	File Medline, abstract 95051568, 1995 XP002124557 & H F SAILER & E KOLB: "Application of purified bone morphogenetic protein (BMP) preparations in cranio-maxillo-facial surgery. Reconstruction in craniofacial malformations and post-traumatic or operative defects of the skull with lyophilized cartilage and BMP" JOURNAL AND CRANIO-MAXILLO-FACIAL SURGERY, vol. 22, no. 4, August 1994 (1994-08), pages 191-199, abstract	1-17
A	File Medline, abstract no. 97195426, 1997 XP002124558 & F H GANNON ET AL.: "Bone morphogenetic protein 2/4 in early fibromatous lesions of fibrodysplasia ossificans progressiva " HUMAN PATHOLOGY, vol. 28, no. 3, March 1997 (1997-03), pages 339-343, abstract	1-17
A	File Medline, abstract no. 89052137, 1989 XP002124559 & P R MAHY & M R URIST: "Experimental hetrotopic bone formation induced by bone morphogenetic protein and recombinant human interleukin-1B" CLINICAL ORTHOPAEDICS AND RELATED RESEARCH, vol. 237, December 1988 (1988-12), pages 236-244, abstract	1-17

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 99/00466

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-4, 6-9, 11, 13-17

Present claims 1-4, 6-9, 11 and 13-17 relate to an extremely large number of possible compounds, methods directed to their manufacture and compositions containing them. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds, preparations and methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the compounds of claims 1 and 8 as further defined by claims 5 and 10, methods directed to their manufacture and preparations containing them.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. ..nal Application No
PCT/IB 99/00466

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9617935 A	13-06-1996	AU 688641 B	12-03-1998
		AU 4120896 A	26-06-1996
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